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Article

No downregulation of immune function during breeding in two year-round breeding bird species in an equatorial East African environment

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Some equatorial environments exhibit substantial within-location variation in environmental conditions throughout the year and yet have year-round breeding birds. This implies that breeding in such systems are potentially unrelated to the variable environmental conditions. By breeding not being influenced by environmental conditions, we become sure that any differences in immune function between breeding and non-breeding birds do not result from environmental variation, therefore allowing for exclusion of the confounding effect of variation in environmental conditions. This create a unique opportunity to test if immune function is down-regulated during reproduction compared to non-breeding periods. We compared the immune function of sympatric male and female chick-feeding and non-breeding red-capped *Calandrella cinerea* and rufous-naped larks *Mirafra africana* in equatorial East Africa. These closely-related species occupy different niches and have different breeding strategies in the same grassland habitat. Red-capped larks prefer areas with short grass or almost bare ground, and breed during low rainfall periods. Rufous-naped larks prefer areas of tall grass and scattered shrubs and breed during high rainfall. We measured the following immune indices: nitric oxide, haptoglobin, agglutination and lysis, and measured total monthly rain, monthly average minimum (T_{min}) and maximum (T_{max}) temperatures. Contrary to our predictions, we found no down-regulation of immune function during breeding; breeding birds had higher nitric oxide than non-breeding ones in both species, while the other three immune indices did not differ between breeding phases. Red-capped larks had higher nitric oxide concentrations than Rufous-naped larks, which in turn had higher haptoglobin levels than red-capped larks. T_{max} was higher during breeding than during non-breeding for red-capped larks only, suggesting potential confounding effect of T_{max} on the comparison of immune function between breeding and non-breeding birds for this species. Overall, we conclude that in the two year-round breeding equatorial larks, immune function is not down-regulated during breeding.

Keywords: agglutination, chick-feeding, environmental conditions, haptoglobin, immune function, larks, lysis, nitric oxide, non-breeding, sympatric species, tropics

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Introduction

Due to the costs associated with its maintenance and expression, immune function has been proposed to be compromised during demanding life cycle events. This is especially during reproduction when animals must allocate resources to production and care of offspring, for example in house sparrow *Passer domesticus* (Bonneaud et al. 2003, Greenman et al. 2005) and tree swallow *Tachycineta bicolor* (Ardia 2005). If life cycle events such as reproduction or migration demand resources that could otherwise be invested in the immune system, the result will be seasonal variation in immune function. Trade-offs between immune function and reproduction have been proposed to be especially manifest in short-and-fast lived species that have evolved a life-history strategy that favours reproduction over self-maintenance. In contrast, long-and-slow lived species are hypothesized to maintain functions that increase survivorship, even under challenging conditions (e.g. reproduction, incremental weather) (Vindervogel et al. 1985, Hughes et al. 1989, Allander and Sundberg 1997, Christe et al. 2000). Although direct evidence from experimental studies for a trade-off between immunity and reproduction is mixed (see Tieleman 2018 for a review), seasonal variation in immune function has nonetheless been reported in multiple studies of temperate and arctic zone birds (Buehler et al. 2008, Martin et al. 2008, Pap et al. 2010a, b, Hegemann et al. 2012, Horrocks et al. 2012). During non-breeding, immune function has been shown to be elevated as individuals are free from reproductive activities that can be energetically and physiologically immunosuppressive (Lee 2006, Martin et al. 2008, Pap et al. 2010a, b).

Because reproduction is restricted to the spring season in temperate and arctic zones, seasonal variation in immune function in these regions could also be explained by seasonally changing environmental conditions. Physiological changes in birds from temperate and arctic zones are mainly driven by day-length (Gwinner 2003, Versteegh et al. 2014), which also plays a major role in determining seasonal changes in environmental factors such as temperature, food availability and pathogen pressure that may also have more direct consequences on immune function of birds. Although some tropical bird species have been shown to use small changes in sunrise and sunset times to regulate annual cycle activities (e.g. stonechats *Saxicola torquatus axillaris* and spotted antbirds *Hylophylax naevioides naevioides*: Dittami and Gwinner 1985, Hau et al. 1998, Hau 2001), some equatorial tropical environments have been referred to as aseasonal, with environmental variation occurring in any month of the year. Alternatively, such regions may experience seasonality that is orchestrated by rainy and dry seasons instead of day-length and temperature (Conway et al. 2005, Ndithia et al. 2017). However, large variation among and within tropical regions makes general characterization of environmental conditions at equatorial latitudes difficult. As a result of generally low variability in day-length and with the occurrence of substantial within-location variation in environmental conditions, many equatorial tropical bird species breed opportunistically and asynchronously, resulting in breeding attempts occurring year round. It is currently unknown if, and how, immune function of such year-round breeding equatorial species varies with reproduction, independent of environmental conditions.

Immune responses can be sensitive to environmental variation (Nelson and Demas 1996, Marra and Holberton 1998, Shepherd and Shek 1998, Ruiz et al. 2002, Tieleman et al. 2005), and sympatric species can differ in their immune responses, for example if they occupy different ecological niches or have different reproductive strategies. In addition, immune function of the same bird species may differ between sexes due to differences in the roles males and females play during reproduction (Sossinka 1980, Emerson and Hess 1996, Møller et al. 2003, Hau et al. 2004) or due to fundamental differences in male and female life-histories (Zuk 1996, Hasselquist 2007, Nunn et al. 2009). Studying males and females of different bird species under the same tropical environmental conditions creates the opportunity to gain a broader perspective on life history trade-offs in tropical birds (Stutchbury and Morton 2008). To our knowledge, no study has yet evaluated the effects of reproduction on immune function, while excluding those of environmental conditions.

Immune function has been hypothesized to vary with the pace-of-life in birds. Whereas temperate and arctic bird species exhibit reduced investment in the immune function and increased investment in reproduction (Ricklefs and Wikelski 2002, Martin et al. 2004, Tieleman et al. 2005), equatorial tropical birds optimize survival (investment in immune defence) over reproduction (Martin et al. 2006, Cox et al. 2010, Previtali et al. 2012) through small clutch sizes. Several studies have demonstrated that longer-and-slower lived bird species favour the maintenance of traits that increase survivorship, such as immune capacity, even under challenging conditions such as reproduction (Tella et al. 2002, Ardia 2005, Lee 2006). To explore if immune function is downregulated during reproduction, while excluding the potential confounding effects of environmental conditions, we exploited a unique study system of year-round breeding by two sympatric tropical bird species, red-capped larks *Calandrella cinerea* and rufous-naped larks *Mirafra africana*, in North Kinangop, Kenya. Our equatorial study location is characterized by large and unpredictable intra-and-inter-annual variations in rainfall (Ndithia et al. 2017). The co-occurrence of the two study species, and their occupation of different niches within the same grassland environment, provides an opportunity for interspecific comparison of reproduction-induced variation in immune function. Our previous study on red-capped larks in three Kenyan locations including North Kinangop revealed that, at the population level, nesting activities in this species fluctuate year-round and are unrelated to rainfall, temperature or invertebrate abundance (Ndithia et al. 2017). Experiencing the same unpredictable intra-and-inter-annual variations in rainfall, Rufous-naped Larks also exhibit

year-round breeding although often not synchronously with Red-capped Larks (HKN unpubl.). We therefore presumed that breeding in Rufous-naped Larks was also unrelated to rainfall, temperature or invertebrate abundance. Since environmental conditions did not influence reproductive decisions (Ndithia et al. 2017), this novel study system enables investigating associations between reproductive activities and immune function.

We asked how the immune function of males and females of red-capped and rufous-naped larks differed between breeding (chick-feeding) and non-breeding birds living in the same equatorial environment that is generally permissive of year-round breeding, and where timing of breeding is not governed by day length, rainfall, temperature or resource availability (Ndithia et al. 2017). We compared immune functions of these two species that live in the same open grasslands, yet occupy different niches within these grasslands and exhibit different reproductive strategies (see Methods for further details). Then, to confirm that any differences in immune function between breeding and non-breeding in the two species do not result from environmental variation, we tested if rainfall, average minimum (T_{min}) and average maximum (T_{max}) temperatures differed between breeding and non-breeding. We expected non-breeding birds to generally have increased investment in immune function and breeding (chick-feeding) ones to have depressed immune function due to expected trade-off between reproduction and immune function (Nelson and Demas 1996, Bentley et al. 1998, Martin et al. 2008). Because we previously did not find any relationship between rain, T_{min} or T_{max} and nesting activity at the population level in North Kinangop (Ndithia et al. 2017), we did not expect these environmental variables to differ between breeding and non-breeding birds in either of the two species.

Material and methods

Study species and study area

Red-capped and rufous-naped larks are sympatric bird species with wide distributions ranging from savannahs with altitudes of 1200 m a.s.l. to highland grasslands 2600 m a.s.l. (Zimmermann 1999). Red-capped lark is a small (mean mass, 25.6 ± 1.54 (SD), n = 66) gregarious bird of short grass to bare ground. Rufous-naped lark is a larger (mean mass, 46.6 ± 4.11 (SD), n = 14) territorial bird that prefers areas with tall grass and scattered shrubs. Both species feed on a variety of invertebrates and occasionally on grass seeds. The two species breed year-round but potentially with different timing of breeding (HKN unpubl.). They build open-cup nests on the ground, often next to a scrub or grass tuft. Mean clutch size is 1.86 ± 0.35 (SD), n = 244 for red-capped larks and 1.81 ± 0.40 (SD), n = 16 for rufous-naped larks. Incubation of eggs lasts between nine and 11 days and the nestling period lasts between nine and 13 d for Red-capped Larks (HKN unpubl.). For Rufous-naped Larks, incubation of eggs lasts between 13 and 15 d while the nestling phase lasts between 10 and 13 d (HKN unpubl.). In both species, only females build the nest and incubate but both sexes feed nestlings. Red-capped larks occurs in large non-territorial flocks when not breeding, but in pairs defending the area around the nest during breeding periods. In contrast, rufous-naped larks defend territories in pairs during breeding and non-breeding periods (HKN unpubl.). Color ring re-sightings suggest that both species are sedentary to our study locations year-round.

We studied both red-capped and rufous-naped larks in three plots in North Kinangop, including Joshua (0°36′00″S, 36°28′27″E, 2451 m ASL), Mbae (0°36′54″S, 36°30′48″E, 2425 m ASL) and Ndarashaini (0°34′33″S, 36°29′41″E, 2412 m ASL). Local variation in soil type, hydrology and rainfall among and within these plots created distinct grassland micro-habitats which the two species occupied and utilised. Red-capped larks preferred the drier parts of the grassland with very short grass, almost bare ground and bred during low rainfall periods. On the other hand, rufous-naped larks preferred the wetter areas with tall grass and scattered shrubs and bred during high rainfall periods. These differences in vegetation and rainfall suggest that these micro-habitats might have the potential to pose different pathogen (micro-organisms and parasites) pressure on the species inhabiting them (Cregger et al. 2012).This might have consequences for the level of investment in immune defence that these two lark species might require. We selected plots based on information from local bird watchers and ourselves about the occurrence of the lark species, and based on provision of permission to access the areas. We worked year-round and simultaneously in these plots from January 2011 to March 2014.

North Kinangop receives on average 584 ± 62.6 (SD) mm of rain per year, and experiences variation in monthly mean T_{min} between 3.0 and 13.7°C, and monthly mean T_{max} between 22.1 and 30.5°C (for details of climatic conditions, see Ndithia et al. (2017). Annual variation in sunrise and sunset times in North Kinangop is less than 35 min (Gwinner and Scheuerlein 1999). Despite some tropical species, e.g. the African stonechat and the spotted Antbird using small changes in sunrise and sunset times to regulate their annual cycle activities (e.g. reproduction, moult) (Dittami and Gwinner 1985, Hau et al. 1998, Hau 2001, Goymann et al. 2012), environmental variation in rainfall and temperature in our study location are independent of calendar month (they are non-seasonal and occur in any month of the year) (Ndithia et al. 2017). Our study species breed year round, opportunistically and asynchronously, and breeding in these species is unrelated to any of the possible proximate factors – rainfall, temperature or food supply (Ndithia et al. 2017).

Field sampling and recording of environmental abiotic variables

We caught non-breeding adult males and females using mist nets and we used cage traps to catch adult males and females at the nest sites during chick feeding. For red-capped larks,

we sampled five and 13 female non-breeding and chick-feeding birds respectively, and 10 males each for non-breeding and chick-feeding. Only one of these birds, a male, was sampled during both non-breeding and chick-feeding. For rufous-naped larks, we sampled four and five female non-breeding and chick-feeding birds respectively, and five and three male non-breeding and chick-feeding birds respectively. We sampled only one male and one female during both non-breeding and chick-feeding. Sampling of the two species partly co-occurred in the same calendar month, and partly occurred in different calendar months, depending on their breeding activities.

From each individual, we collected a blood sample for immune function analyses using heparinized capillary tubes after carefully puncturing the brachial vein on the wing. We put blood samples in eppendorf tubes, temporarily stored them in ice and centrifuged them at the end of each fieldwork day. We stored the plasma fraction in the freezer (−20°C) for future analyses. To obtain total monthly rainfall (mm), monthly average minimum (T_{min}) and monthly average maximum (T_{max}) temperatures (°C), we set up a weather station (Alecto WS-3500, Den Bosch, Netherlands) in a secure location placed centrally to the three field sites. Direct distances from weather station locations to field sites were as follows: 3.8 km to Joshua; 2.5 km to Mbae and 1.8 km to Ndarashaini (Ndithia et al. 2017).

Immune assays

Haptoglobin (mg ml^{-1}) is an acute phase protein that scavenges haemoglobin in the event of haemolysis and increases several-fold in the event of infection, injury or malignancy (Quaye 2008). We determined haptoglobin concentration using an assay that measures the haem-binding capacity of plasma (TP801; Tridelta Development limited, Maynooth, Ireland) following instructions provided by the manufacturer and with incubation at 30°C for 5 min following Matson et al. (2012). Each of the three assay plates, included an among-plate standard which we ran in duplicate within each plate (Matson et al. 2012) (mean within-plate coefficient of variation (CV) = 2.4%; mean among-plate CV = 2.7%).

Nitric oxide (mmol ml^{-1}) is a multifunctional signalling molecule that, among others, modulates inflammatory processes and participates in destroying parasites, virus-infected cells and tumor cells, providing information about animal condition (Sild and Hõrak 2009). We determined nitric oxide production through the reduction of nitrate to nitrite by copper-coated cadmium granules, followed by color development with Griess reagent (Promega; Sild and Hõrak 2009) and absorbance measurement at 542 nm (Versamax, Molecular Devices Sunnyvale, California, USA) (Sild and Hõrak 2009).

Complement (hemolysis) and natural antibodies (hemagglutination) are constitutively present in the innate immune system (Matson et al. 2005). We quantified complement lysis titres and natural antibody agglutination titres against red blood cells of rabbit (Envigo, Belton, UK) through serially diluting plasma samples according to the assay of Matson et al. (2005). Lysis indicates the interaction of complement and natural antibodies. Agglutination reflects the interaction between natural antibodies and antigens of rabbit red blood cells. We scored hemolysis and hemagglutination titres blind to sample and plate identity at least twice. We used the mean in the analyses if the first two scores were less than one titre apart. If the difference of the first two scores was more than one, we scored a third time and used the median in analyses (Matson et al. 2005). We assigned half scores to samples that showed intermediate lysis and agglutination. We calculated among-plate and within-plate variation for lysis (mean among-plate CV = 18.6%; mean within-plate CV = 9.8%), and for agglutination (mean among-plate CV = 9.7%; mean within-plate CV = 7.7%).

Statistical analyses

We used generalized linear models (glm) with normal (Gaussian) distribution for analyses of haptoglobin, nitric oxide and agglutination, and with binomial distribution for analysis of lysis. Although perhaps ideal, using a mixed-effects model with either bird ID or nest ID as random factor was precluded by the design of the data set: we sampled different individuals during breeding and non-breeding, while during breeding we mostly sampled males and females that attended different nests (all individuals for rufous-naped larks, and 40% of the individuals for red-capped larks). To test for differences in immune function and mass (g) between breeding and non-breeding in different sexes of red-capped and rufous-naped larks, we constructed a model of each immune index (haptoglobin, nitric oxide, agglutination and lysis) as dependent variables and with explanatory variables breeding status, species, sex and all three-way and two-way interactions. We square-root transformed data of haptoglobin and log-transformed data of nitric oxide to obtain normality because the residuals of models for these two indices were not normally distributed.

The haptoglobin assay may be affected by plasma sample redness due to sample hemolysis (Matson et al. 2012). During the assay, we ran a 450 nm pre-scan to enable us to statistically correct for plasma sample redness. In addition, plasma sample age (range in sample age: 82–1256 d) possibly affects immune assays involving haptoglobin, nitric oxide, agglutination and lysis. In cases where plasma sample redness significantly affected haptoglobin and where plasma sample age significantly affected any of the immune indices, we included them in the respective models as a covariate. Haptoglobin was affected by plasma sample age ($F_{1,49} = 9.78$, $p = 0.003$) and plasma sample redness ($F_{1,49} = 10.49$, $p = 0.002$), and plasma sample age affected nitric oxide ($F_{1,46} = 5.88$, $p = 0.02$), agglutination ($F_{1,48} = 8.12$, $p = 0.01$) and lysis ($\chi^2 = 5.07$, $df = 1$, $p = 0.02$). We tested the effect of breeding status on mass for both lark species using two separate models because rufous-naped larks are almost twice the mass of red-capped larks. Each model included mass as dependent variable and explanatory variables breeding status, sex and their interaction.

To check whether environmental conditions confound the effect(s) of breeding on immunity, we tested if environmental conditions (rain, T_{min} and T_{max}) differed during chick-feeding and non-breeding periods in males and females of the two species by matching the date (month) of the immune measurement of each individual bird with the corresponding total monthly rainfall, T_{min} and T_{max}. Using rain, T_{min} and T_{max} as dependent variables, we build models that included explanatory variables breeding status, species, sex and all three-way and two-way interactions. However, we did not include environmental variables in models that also included measures of immune indices. A model including environmental variables, breeding status, sex, species and immune indices would require sufficient spread of immune function data for environmental conditions representing the breeding and non-breeding data sets for both species, which our current data sets lack.

We used type III sum of squares in the anova summary of results to test main effects in the light of interaction terms as well as in the light of other main effects (Mangiafico 2015). Whenever an interaction was significant, we made a new variable consisting of all the separate variables in the interaction and ran a Tukey's post hoc test on this new variable. For all analyses, we tested and confirmed that the residuals of the final models observed the assumptions of normality and homoscedasticity of variance through graphical and statistical methods. We simplified models using backward elimination by deleting the least significant terms from the model until we arrived at the final model and used $p < 0.05$ as selection criterion. The final model consisted of all the significant terms, any of the non-significant main effects of breeding status, species and sex and co-variates (sample age, sample redness) if applicable. We used R statistical software (ver. 3.0.3) (R Core Team) in all our analyses.

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.9rt5dd8> (Ndithia et al. 2019).

Results

Immune function and body mass during breeding and non-breeding

We found no uniform differences between chick-feeding and non-breeding larks for the four immune indices, but some indices did vary with breeding status and between species and sexes (Fig. 1A–D, Table 1). We found significant effects of breeding status and species for nitric oxide, a significant effect of species on haptoglobin and a significant three-way interaction of breeding status × species × sex for agglutination (Fig. 1A–C, Table 1). Chick-feeding birds had significantly higher nitric oxide than those that were not breeding in both species, while red-capped larks had significantly higher nitric oxide than rufous-naped larks (Fig. 1A). Conversely, rufous-naped larks had significantly higher haptoglobin than red-capped larks (Fig. 1B). Post-hoc tests to further explore the significant three-way interaction breeding status × species × sex for agglutination only revealed that non-breeding females had higher agglutination than non-breeding males in red-capped lark ($t = 3.39$, $p = 0.02$, Fig. 1C); all other pairwise comparisons were non-significant (all $p > 0.18$). We did not find an effect of breeding status on haptoglobin or effects of breeding status and species on lysis, while nitric oxide, haptoglobin and lysis did not differ significantly among sexes (Fig. 1A, B, D, Table 1). Body mass tended to be lower during chick-feeding than during non-breeding in red-capped larks males and females and in rufous-naped larks females, but not in rufous-naped larks males (Fig. 1E–F). Breeding status had a marginally non-significant effect on body mass in red-capped larks, while sex had no effect (Fig. 1E, Table 1). In contrast, body mass in rufous-naped larks did not significantly differ between breeding and non-breeding, but it did with sex, since males were significantly heavier than females (Fig. 1F, Table 1).

Rainfall and temperature during breeding and non-breeding

Red-capped and rufous-naped larks appeared to experience differences in rainfall, T_{min} and T_{max} between chick-feeding and non-breeding, despite living in the same environment (Fig. 2). Remarkably, patterns were opposite in the two lark species: red-capped larks appeared to experience relatively low rainfall, low T_{min} and high T_{max} when they fed chicks compared to when they did not breed. In contrast, rufous-naped larks appeared to experience higher rainfall, higher T_{min} and lower T_{max} when they were feeding chicks than when not breeding (Fig. 2). When statistically testing these patterns, the interaction breeding status × species was significant for rain and T_{max} but not for T_{min} which was marginally non-significant and for which the main effects were also not significant (Table 2). Subsequent post-hoc tests revealed that rainfall was not significantly lower during chick-feeding compared to when not breeding in both species (red-capped larks $t = 2.35$, $p = 0.08$; rufous-naped larks $t = 1.69$, $p = 0.29$). Although insignificant, rainfall tended to be higher when red-capped larks were not breeding than when rufous-naped larks were not breeding ($t = 2.31$, $p = 0.08$), while the difference in rainfall was non-significant when the two species were feeding chicks ($t = 1.57$, $p = 0.34$). Post hoc tests further showed that red-capped larks fed chicks at significantly higher T_{max} than when they were not breeding ($t = 4.50$, $p < 0.001$), but T_{max} did not differ between chick-feeding and non-breeding periods in rufous-naped larks ($t = 0.68$, $p = 0.88$). Red-capped larks fed chicks at significantly higher T_{max} than rufous-naped larks ($t = 2.77$, $p = 0.03$), but T_{max} did not differ between the two species when they were not breeding ($t = 1.73$, $p = 0.27$).

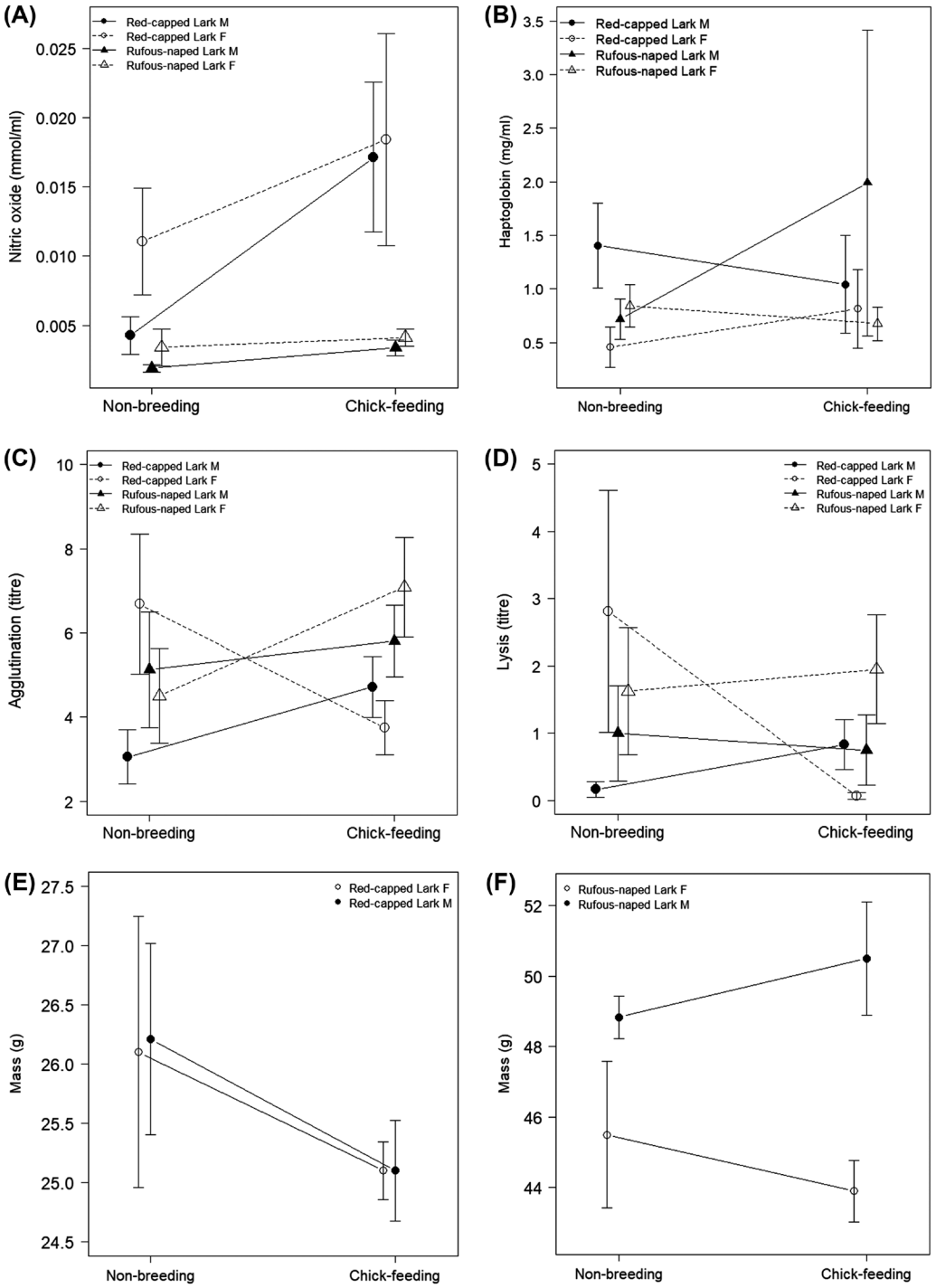

Figure 1. (A) Nitric oxide (mean ± SE, mmol ml^{-1}), (B) Haptoglobin (mean ± SE, mg ml^{-1}), (C) agglutination (mean ± SE, titre), (D) Lysis (mean ± SE, titre), (E and F) mass (g) in chick-feeding and non-breeding red-capped larks *Calandrella cinerea* and rufous-naped larks *Mirafra africana* in North Kinangop, Kenya.

Discussion

Studying four immune indices in two sympatric bird species, red-capped and rufous-naped larks in equatorial East Africa, we found that haptoglobin, agglutination and lysis did not differ between breeding and non-breeding, but nitric oxide did, although contrary to prediction; chick-feeding birds had higher nitric oxide than those that were not breeding. Although sex did not affect any of the immune indices, there was high variation in the pattern of all immune indices in both species (except nitric oxide), suggesting that immune function of different sexes responded to differences in

Table 1. Results of models examining variation in immune function between chick-feeding and non-breeding male and female red-capped larks *Calandrella cinerea* and rufous-naped larks *Mirafra africana* in North Kinangop, Kenya. Nitric oxide data was log-transformed and haptoglobin data was square root transformed to obtain normality. p values < 0.05 are indicated in bold.

Variable	Explanatory variable	df	F	p
Nitric oxide (mmol ml^{-1})	breeding status × species × sex	1,39	0.34	0.56
	species × sex	1,40	0.15	0.70
	breeding status × species	1,41	0.43	0.51
	breeding status × sex	1,42	2.84	0.10
	sex	1,43	1.34	0.25
	breeding status	1,44	5.17	**0.03**
	species	1,44	8.69	**0.005**
Haptoglobin (mg ml^{-1})	breeding status × species × sex	1,41	0.65	0.43
	breeding status × species	1,42	0.46	0.50
	breeding status × sex	1,43	0.62	0.44
	species × sex	1,44	2.70	0.11
	breeding status	1,45	1.82	0.18
	species	1,45	4.85	**0.03**
	sex	1,45	2.82	0.10
Agglutination (titre)	breeding status × species × sex	1,41	6.46	**0.01**
Lysis (titre)	breeding status × species × sex	1,41	1.34	0.25
	breeding status × species	1,42	0.006	0.94
	species × sex	1,43	0.37	0.54
	breeding status × sex	1,44	2.82	0.09
	sex	1,45	0.10	0.76
	species	1,45	0.14	0.71
	breeding status	1,45	0.25	0.62
Mass (g), red-capped lark	breeding status × sex	1,35	0.01	0.93
	sex	1,36	0.01	0.94
	breeding status	1,36	3.37	0.07
Mass (g), rufous-napped lark	breeding status × sex	1,11	1.25	0.29
	breeding status	1,12	0.04	0.85
	sex	1,12	11.65	**0.005**

reproductive roles of males and females, or due to differences in life-histories. Body mass of rufous-naped larks also differed between males and females suggesting different effects of reproduction on males and females. Nitric oxide and haptoglobin differed between species with red-capped larks having higher nitric oxide than rufous-naped larks, which in turn had higher haptoglobin than red-capped larks, suggesting differences in life history adaptations of sympatric species facing variable and unpredictable environmental conditions. Non-breeding females had higher agglutination than non-breeding males in red-capped larks, the only immune index affected by sex. Body mass did not differ between breeding and non-breeding in any of the two species. Sex had an effect on body mass only in rufous-naped larks, with heavier males than females. The environmental data confirmed that rainfall and T_{min} did not differ between breeding and non-breeding birds for both species. This was also the case for T_{max} for rufous-naped larks, but for red-capped larks T_{max} was higher during chick-feeding than during non-breeding. Hence, for red-capped larks we cannot fully rule out a confounding effect of environmental conditions (i.e. T_{max}) on the comparison of immune function between breeding and non-breeding birds. Overall, we conclude that two tropical larks do not downregulate immune function during breeding. We propose that a productive future step would be to study if and how the highly variable environmental conditions shape variation in immune function in this system.

We had expected chick-feeding birds to display depressed immune function due to the expected trade-off between reproduction and immune function. However, our results indicated that chick-feeding birds of both species had higher nitric oxide than those that were not breeding, while the other immune indexes did not differ between breeding and non-breeding. This suggests that these two species have the capacity to maintain both reproduction and immune function simultaneously without adjustment of either. This is in line with other studies that show that immune function varies with the pace-of-life (Martin et al. 2006, Cox et al. 2010, Previtali et al. 2012). We can conclude that the hypothesized immunosuppression due to the cost of reproduction is not generally applicable to all birds. Our findings could be in support of the more nuanced hypothesis that immunosuppressive costs of reproduction are more manifest in short-and-fast lived species than in long-and-slow lived birds. Although we have no data on the life expectancy of these lark species, tropical birds are generally thought to be longer-and-slower lived with well-developed immune defences (Martin et al. 2006, Lee et al. 2008). Hence it may not be surprising that these tropical larks follow a strategy that favours the maintenance of functions that increase survivorship such as immune

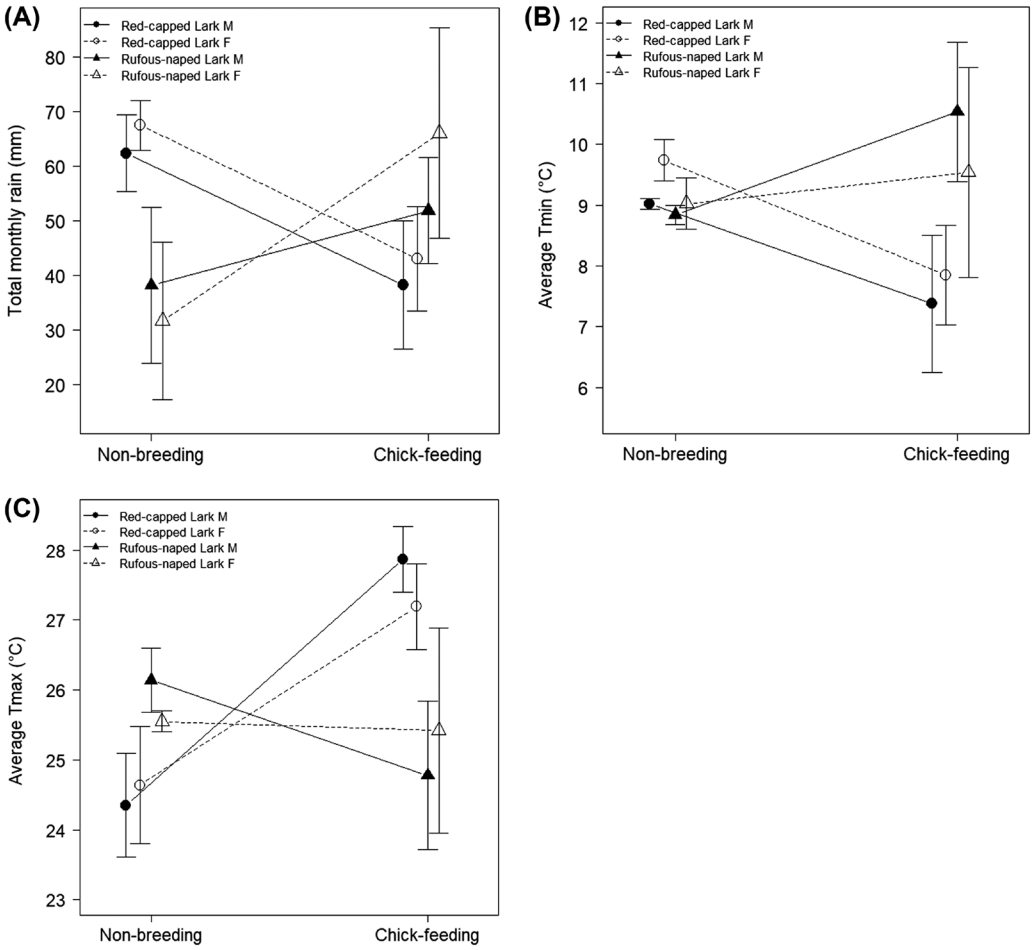

Figure 2. Relationships between (A) = rain, (B) average minimum temperature (T_{min}), (C) average maximum temperature (T_{max}) and the different breeding statuses of male and female red-capped larks *Calandrella cinerea* and rufous-naped larks *Mirafra africana* in North Kinangop, Kenya.

Table 2. Results of models testing relationships between abiotic environmental factors (rain, mm) average minimum temperature (T_{min}, °C) and average maximum temperature (T_{max}, °C) and chick-feeding and non-breeding male and female red-capped larks *Calandrella cinerea* and rufous-naped larks *Mirafra africana* in North Kinangop, Kenya. Significant p values < 0.05 are in bold.

Environmental variable	Explanatory variable	df	F	p
Rain (mm)	breeding status × species × sex	1,46	0.32	0.58
	species × sex	1,47	0.0021	0.96
	breeding status × sex	1,48	0.15	0.70
	sex	1,49	0.26	0.61
	breeding status × species	1,49	7.59	**0.01**
T_{min} (°C)	breeding status × species × sex	1,46	0.10	0.75
	breeding status × sex	1,47	0.17	0.69
	species × sex	1,48	0.42	0.52
	breeding status × species	1,49	3.51	0.07
	breeding status	1,50	1.43	0.23
	species	1,50	1.83	0.18
	sex	1,50	0.11	0.74
T_{max} (°C)	breeding status × species × sex	1,46	0.79	0.38
	species × sex	1,47	0.04	0.85
	breeding status × sex	1,48	0.06	0.81
	sex	1,49	0.15	0.70
	breeding status × species	1,49	10.06	**0.003**

capacity even under challenging conditions such as reproduction (Tella et al. 2002, Ardia 2005, Lee 2006, Lee et al. 2008). Several other studies have also demonstrated immunocompetence in equatorial tropical birds even during reproduction (Vindervogel et al. 1985, Hughes et al. 1989, Allander and Sundberg 1997, Christe et al. 2000). This strategy may in fact be less demanding because of the relatively small clutches that these larks lay (both species have a clutch size of two (see Methods) (Deerenberg et al. 1997, Moreno et al. 1999, Hanssen et al. 2005) compared to for example, temperate skylarks *Alauda arvensis* (mean = 3.53 + 0.43, SE, range, 3–5, n = 33) (Delius 1965, Wilson et al. 1997) and woodlarks *Lullula arborea* (mean = 4.05 + 0.06, SE) (Wright et al. 2009). The elevation instead of downregulation of nitric oxide could mean that breeding individuals are more immunocompetent and/or less challenged than non-breeding ones. An alternative explanation is that challenged females omit breeding, automatically selecting only for birds with low nitric oxide in our sample. Both explanations would be in line with a long life expectancy.

In the case of the red-capped larks, the elevated nitric oxide during breeding coincided with higher T_{max}. This co-occurrence raises the possibility that high T_{max} provided a conducive environment for growth, development and reproduction of microorganisms and parasites (Sehgal et al. 2011, Zamora-Vilchis et al. 2012), and that birds responded to this with elevation of nitrogen oxide. Immune function in birds is known to vary based on prevailing environmental conditions in tropical, e.g. weak immune function in superb starlings *Lamprotornis superbus* during dry (low rainfall conditions) (Rubenstein et al. 2008), in desert versus temperate zone where variation in immune function is associated with differences in antigen exposure in larks living in a gradient of environmental variation (Horrocks et al. 2012), better immune response with rainfall in temperate house martin *Delichon urbica* (Christe et al. 2001) and seasonal variation in temperate skylarks *Alauda arvensis* (Hegemann et al. 2012). Birds increase their immune function due to abundance of parasites and microbes in their environments (Christe et al. 2001, Møller et al. 2003, Horrocks et al. 2012).

Red-capped larks had relatively high nitric oxide and low haptoglobin compared with rufous-naped larks while the two species live in the same environment. This either suggests within-location variability in exposure to disease and parasites, interspecific differences in susceptibility or interspecific differences in immune strategies to deal with the same problem. Although the two species generally consume similar diets and have access to similar amounts of food, they occupy different niches within these grasslands (see Methods for details of these differences). High variability in patterns of rainfall within North Kinangop tends to create distinct micro-habitats occupied by the two species with potentially different vulnerabilities. Microbial communities and parasites can vary in space even within a population (Bensch and Åkesson 2003, Angel et al. 2010, Froeschke et al. 2010, Knowles et al. 2010). Other studies comparing birds living in the same environment have also found that some species are more susceptible to disease than others (Perkins and Swayne 2003, Tumpey et al. 2004). In addition, the immune function of different species may respond differently to the same parasite or microbial infection in their environment (Blount et al. 2003, Matson et al. 2005, Pap et al. 2010a, b), and immune function may also differ among species due to body size differences (Hasselquist 2007).

Females had higher agglutination than males in red-capped larks during non-breeding periods. Females frequently display more robust immune response than males owing to either the different roles of the sexes or to fundamental differences in male and female life-histories (Zuk 1996, Hasselquist 2007, Nunn et al. 2009). In our earlier study, we proposed that red-capped larks may be an opportunistic breeder, breeding whenever environmental conditions are permissive (Ndithia et al. 2017). Opportunistically breeding males may invest in maintaining their reproductive capacity all year round in order to respond quickly to favourable reproductive conditions (Sossinka 1980, Emerson and Hess 1996, Hau et al. 2004), in which case the relatively high levels of reproductive hormones in males may suppress the immune function (Møller et al. 2003).

Our study system exemplifies the high variability in environmental conditions in equatorial tropics and how sympatric bird species living under these conditions utilize them differently to presumably maximize reproductive success and optimize their protection against diseases and parasites. Our study highlights the stark differences between temperate and equatorial tropical birds in in light of the timing of life cycle events such as reproduction. It supports the proposition that equatorial tropical birds, exhibiting a slow pace-of-life strategy, optimise survival (investment in immune function) over reproduction, as exemplified by their relatively smaller clutch sizes. Further studies are needed to investigate the temporal patterns of pathogen and parasite pressures year-round, and to investigate the potential role of environmental conditions, particularly T_{max}, in the temporal variation of immune function. It will then be necessary to link any such temporal variation with a fuller study of year-round variation in immune function, to understand how the changing suite of immune defences is matched to the changing landscape of threats. An interesting further study topic would to be to investigate variation in immune function on individuals during the year and during different breeding events.

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Author contributions – BIT conceived this study. BIT and HKN designed and raised funds for the study. HKN was responsible for project administration and collection of most field data with supervision of BIT; BIT and MAV helped with intensive data collection for one month in a year for 3.5 years. MM visited field study sites, gave insights into data collection and supported HKN through field data collection and in seeking permits to field locations. MAV and HKN (including Dr. Kevin Matson) analysed plasma samples for four immunological indices in the laboratory using assay protocols. MAV advised on data analyses. HKN analyzed and interpreted the data, and wrote the manuscript on which MAV and MM gave comments. BIT supervised data analyses and interpretation and manuscript writing. All authors read and approved the contents of the manuscript for publication.
Permits – We followed all applicable institution guidelines for the care and use of animals. The National Museums of Kenya, a state corporation with a research mandate, and being a national research institution with well-trained and qualified personnel in animal handling, approved this research through a letter by the Human Resource Training and Development Committee, Ref: NMK/TRN/PF/177015/045. Neither of the study species involved are an endangered or protected species. We (HKN, MAV, BIT) the project team involved in capture and handling of birds, are all skilled bird ringers; we captured and handled birds in adherence to animal welfare as stipulated in the standard operating protocols of the National Museums of Kenya.

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